Chlamydia pneumoniae antigens facilitate experimental aortic dilatation: Prevention with azithromycin

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Objective: **The purpose of this study was to investigate whether *Chlamydia pneumoniae* (live, antigens, or polysaccharide) cause abdominal aortic aneurysm in a susceptible animal host with appropriate drug reversal.**
Methods: **At laparotomy, preparations of *C pneumoniae* (live, formalin-inactivated, and heat-inactivated) in calcium chloride were applied to the adventitial surface of the abdominal aorta of rabbits fed a cholesterol-enriched diet. Aortic diameter was measured with ultrasonography. After 3 weeks, immunohistochemistry was used to detect aortic *C pneumoniae* and macrophages. Presence of *C pneumoniae* DNA also was assessed.**
Results: **At high doses (5×10^7organisms) periaortic application of both live and formalin-inactivated preparations resulted in doubling of aortic diameter after 3 weeks, from 2.0 ± 0.5 mm to 4.3 ± 1.3 mm ($P < .02$). *C pneumoniae* DNA and antigens, together with a heavy macrophage infiltrate, were detected in the dilated aorta. In contrast, periaortic application of heat-inactivated preparations resulted in minimal macrophage influx and aortic dilatation. Treatment of rabbits with azithromycin or carprofen for 10 days after laparotomy abolished the effects of formalin-inactivated *C pneumoniae* on aortic dilatation. Azithromycin reduced the number of macrophages in the aortic wall more effectively than carprofen.**
Conclusion: **Because membrane antigenicity is retained in formalin-inactivated but not heat-inactivated organisms, in this experimental model, chlamydial membrane antigens (rather than live organisms) appear to cause the aneurysmal dilatation and associated macrophage recruitment. Azithromycin is likely to reverse these effects with an antiinflammatory mechanism. (J Vasc Surg 2002;36:1011-7.)**

At the beginning of the 20th century, Sir William Osler proposed that infectious agents could be important in the pathogenesis of atherosclerosis. At the beginning of the 21st century, the association between atherosclerosis and infection with *Chlamydia pneumoniae* is accepted widely. Most of this evidence has been seroepidemiologic[1-4] or pathologic.[4-6] Cell biology has revealed some of the potential molecular mechanisms whereby infection with *C pneumoniae* might cause or accelerate atherosclerotic disease. Such mechanisms include the ability of the chlamydial heat shock protein to stimulate cytokine and metalloproteinase secretion from macrophages and the increased procoagulant functions of endothelium and smooth muscle cells after infection with *C pneumoniae*.[7,8]

Abdominal aortic aneurysm (AAA) is a common condition in men more than 65 years of age. Traditionally, AAA was viewed as a complication of atherosclerosis.[9] Today, the crucial pathologic processes underlying the degeneration of the aortic media in aneurysms appear to be inflammation and proteolysis.[10] There is also a link with *C pneumoniae* infection. In a small study, Juvonen and colleagues[11] showed that more than half of patients with AAA had a high serum antibody immunoglobulin G (IgG) titre, an even larger proportion showed evidence of chlamydial elementary bodies or antigens in aortic wall biopsies, and all biopsies assessed for the presence of chlamydial DNA tested positive. This has been followed by further studies, principally with use of the polymerase chain reaction to detect DNA, which showed evidence of chlamydial infection in 50% to 100% of aortic biopsies.[12-14] No evidence indicates whether the association between the presence of *C pneumoniae* and AAA is casual or causal.

Traditionally, where an infectious agent causes a specific disease, all three of Koch's postulates must be satisfied. The first two of these postulates have been satisfied for the association between *C pneumoniae* and atherosclerosis: the organism is found in association with the disease, and the microorganism can be isolated and grown in pure culture.[15] The third postulate, that disease can be reproduced in a susceptible host (animal) with inoculation with the organism, has been more difficult to prove. Some evidence

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exists that high doses of *C pneumoniae*, administered intranasally, accelerate intimal thickening and the development of atherosclerosis in cholesterol-fed rabbits.[16] Treatment of the rabbits with azithromycin prevented the accelerated intimal thickening.[16]

In man, the causative role of *C pneumoniae* has been and continues to be addressed through antibiotic trials for the secondary prevention of coronary events. These trials have had mixed results, and currently, antibiotic therapy to reduce vascular risk appears inappropriate.[17,18] A pilot trial in 34 patients has indicated that doxycycline may reduce the growth rate of small AAAs, although no effect was seen on chlamdial antibody titres.[19]

In this study, we have tried to satisfy Koch's third postulate for AAA. Like previous workers, we have used rabbits fed a cholesterol-enriched diet. In these animals, the periaortic application of calcium chloride and thioglycollate (a nonspecific macrophage activator) resulted in a two-fold increase in aortic diameter within 3 weeks, although at this time point, rupture was only observed in approximately 2% of cases.[20] The calcium chloride causes endothelial dysfunction, increased aortic permeability, and calcification of the media; thioglycollate is a nonspecific macrophage stimulant, and in rabbits, hypercholesterolemia increases macrophage activity.[20] We have replaced the thioglycollate with either live *C pneumoniae*, formalin-inactivated preparations (with preservation of antigenicity[7]), or heat-inactivated preparations (with preservation of lipopolysaccharide but not antigenicity[7]) and investigated the effects on aortic diameter and macrophage influx.

METHODS

New Zealand white rabbits (2.2 to 2.8 kg) were subject to laparotomy in accordance with approved protocols for the care and use of laboratory animals (PIL70/4221). The rabbits were fed either a standard chow or a cholesterol-enriched diet (Stanrab + 0.5% cholesterol; Lillico, Betchworth, United Kingdom) starting 2 days before surgery: all rabbits initially were seronegative for chlamydial antigens. Anesthesia was obtained with a combination of intramuscular fentanyl citrate (Hypnorm, Roche, United Kingdom) and intravenous midazolam (Hypnovel, Janssen, United Kingdom), with oxygen at 2 L/min delivered with facial mask. After fentanyl citrate injection, before laparotomy, the diameter of the abdominal aorta (mean of three anterior-posterior readings) was measured with ultrasonography (Aloka SSD-500 with 7.5-MHz probe; Keymed, Southend, United Kingdom), limits of agreement ± 0.2 mm.[20] The abdomen was opened through a midline incision, and the infrarenal aorta was dissected, with care not to damage any lumbar vessels. Calcium chloride solutions (0.2 mol/L) were applied at a slightly lower concentration than described previously[20] to avoid excessive aortic calcification. *C pneumoniae* were applied, to the infrarenal aorta, in the calcium chloride solution (total volume, 4.0 mL, containing 5×10^1 to 5×10^7 organisms, of which 10% to 15%, by weight, were retained on the applicators). The chlamydial preparations (live, formalin-inactivated, and heat-inactivated) were a kind gift from Dr Brenda Thomas (Imperial College at St Mary's, London); heat and formalin-inactivated preparations had no replicative activity in culture. Similarly, sodium thioglycollate (0.1 mol/L) was applied in 3.5 to 4.0 mL of calcium choride solution. The abdomen was sutured with mass closure with 3/0 prolene, followed by subcuticular skin suture (3/0 Dexon, Norrtalje, Sweden). Some rabbits were treated with oral azithromycin (4 mg before surgery, followed by 4 mg/d for 10 days after surgery) or subcutaneous carprofen (10 mg before surgery and on the first postoperative day followed by 5 mg/d for 10 days). Three weeks later, the diameter of the aorta was again measured with ultrasonography, after fentanyl citrate injection, a serum sample removed for the measurement of cholesterol (with a colorimetric enzyme assay kit, Boehringer, Lewes, United Kingdom), and the animals killed. Some animals had the aorta and other vessels fixed with perfusion with 5% glutaraldehyde at a pressure of 100 cm water. Where the aorta was not perfusion fixed, a sample was frozen at −70° C for later extraction of DNA and the remainder fixed in formalin.

All aortas were taken in cross section and processed with hematoxylin and eosin and elastic van Gieson staining. The stained sections were assessed by a single consultant histopathologist. Macrophages were further identified with the antibody RAM-11 (Dako, Ely, United Kingdom) at 1 in 50 dilution and elemental bodies of *C pneumoniae* with the fluoroscein isothiocyanate conjugated monoclonal antibody TT-401 (Washington Research Foundation, Seattle, Wash) at 1 in 50 dilution. Stained macrophages were counted by a single observer, blind to the treatments applied to each specimen. For quantitative analysis, cells from consecutive, circumferential, nonoverlapping aortic fields were examined under a digital image capturing light microscope and cell counting performed with AnalySiS (Perceptive Instruments, Steeple Bumpstead, United Kingdom) image analysis software. Aortic wall thickness (lumen to external elastic lamina) was measured in four quadrants, and the mean was calculated.

DNA was extracted from frozen pulverized tissue with a commercial kit (Dneasy, Qiagen, Crawley, United Kingdom). Amplification of the omp-1 and β-actin genes was performed with 50 to 100 ng of template DNA, in a 50-μL reaction mixture containing 1.5 to 2.5 mmol/L $MgCl_2$, 2 U Taq (Promega, Southampton, United Kingdom) polymerase, 10 pmol primers (Perkin-Elmer, Warrington, United Kingdom), 1 volume buffer "B" (Promega, Southampton, United Kingdom). and 200 μmol/L deoxynucleotide triphosphates (Pierce & Warriner, Chester, United Kingdom. The β-actin primers (5'-3') were ATCATGTTTGAGACCTTCAACACCCC and CTTGATCTTCATTGTGCTGGGTGCCA. If the β-actin gene could not be amplified from the DNA sample, it was subject to further phenol-chloroform extraction and ethanol precipitation. The first and second round primers (5'-3') for amplification of the omp-1 gene were GTTGTTCATGAAGGCCTACT, TGCATAACCTACGGTTT

Effect of different *C pneumoniae* preparations on aortic diameter, 3 weeks after periaortic application

	n	*Mean aortic diameter with ultrasonography: initial (mm)*	*Mean aortic diameter with ultrasonography: final (mm)*	*TT-401 staining at 3 weeks*
Added to 0.2 mol/L $CaCl_2$	4	2.8 ± 0.4	3.0 ± 0.5	0/4
0.1 mol/L thioglycollate	6	2.8 ± 0.4	4.8 ± 0.4*	nd
C pneumoniae live	4	2.0 ± 0.5	4.3 ± 1.3*	4/4
C pneumoniae heat-inactivated	5	2.4 ± 0.2	3.5 ± 0.5	0/5
C pneumoniae formalin-inactivated	7	2.4 ± 0.7	4.3 ± 0.6*	5/5

Chlamydial doses were 5×10^7 organisms.
Results are given as mean ± standard deviation.
*Significant increase in diameter compared with initial diameter (paired *t* test, $P < .02$).
nd, Not determined.

and TTTAGATCATGGTGTCATTCGC, AAGGTTCATCCTTGAAGGCA, respectively. This nested reaction can be sensitive to polymerase chain reaction (PCR) inhibition,[5] so that if no amplification was achieved, the DNA was diluted 10-fold and spiked and tested in the presence and absence of cloned TWAR DNA. Cloned TWAR183 DNA was the kind gift of Dr Nessa Carey, Imperial College at Charing Cross, United Kingdom, and the sensitivity of the nested omp-1 gene amplification was 70 gene copies.

Most data were analysed with analysis of variance (ANOVA), giving Fischer exact significance for paired comparisons. Aortic diameters were compared with paired *t* tests. Numerical values are reported as mean ± standard deviation, unless otherwise stated.

RESULTS

Effect of *C pneumoniae* on aortic diameter. In rabbits fed a cholesterol-enriched diet, periaortic application of 0.2 mol/L $CaCl_2$ did not result in an increase in aortic diameter after 3 weeks (negative control; Table). After 3 weeks, the average weight gain of rabbits was 62 g and the median serum cholesterol concentration was 72 mmol/L. Periaortic application of 0.2 mol/L $CaCl_2$ with 0.1 mol/L thioglycollate resulted in a two-fold increase in aortic diameter after 3 weeks (positive control; Table). Periaortic application of 0.2 mol/L $CaCl_2$ containing 5×10^7 organisms of live *C pneumoniae* resulted in a two-fold increase in aortic diameter after 3 weeks (Table). With the same dose of organism, we then compared the effect of live, formalin-inactivated, and heat-inactivated *C pneumoniae* on aortic dilatation and macrophage influx. Periaortic application of formalin-inactivated *C pneumoniae* (5×10^7 organisms) in 0.2 mol/L $CaCl_2$ also resulted in a two-fold increase in aortic diameter at 3 weeks, but application of heat-inativated *C pneumoniae* had no significant effect on aortic diameter (Table). Lower doses of formalin-inactivated *C pneumoniae* ($5 \times 10^1$to 5×10^5 organisms) did not have a significant effect on aortic diameter at 3 weeks (data not shown).

Persistence of *C pneumoniae* in postmortem aorta. The presence of the omp-1 gene (with PCR) and elemental bodies (TT-401 immunofluorescence) 3 weeks after periaortic application of chlamydia preparations were assessed. For aortas treated with live *C pneumoniae* (5×10^7 organisms), the omp-1 gene was amplified successfully from two; in two further aortas, there was evidence of PCR inhibition, and all samples showed positive staining for elemental bodies with the monoclonal antibody TT-401. For five rabbits treated with the full dose of formalin-inactivated *C pneumoniae*, the omp-1 gene was successfully amplified from two aortas; the remaining three showed evidence of PCR inhibition, but all five showed positive staining with TT-401 (Fig 1). In contrast, for five rabbits treated with heat-inactivated *C pneumoniae*, there was no evidence of either the omp-1 gene or PCR inhibition and none of the aortas stained positively with TT-401 (a summary of the TT-401 staining results is given in the Table). However, the omp-1 gene could be amplified from splenic DNA from these latter animals. The omp-1 gene also could be amplified from undilated aortas, where formalin-inactivated *C pneumoniae* (5×10^7 organisms) had been applied in NaCl rather than $CaCl_2$ or where rabbits had not received a high cholesterol diet. Therefore, there was DNA and immunohistochemical evidence for aortic retention of both live and formalin-inactivated organisms but not for the heat-inactivated organisms.

Inflammatory infiltrates and macrophage recruitment. After 3 weeks of cholesterol feeding, there were only scant numbers of macrophages (6 ± 6 per unit area) and few other inflammatory cells in the abdominal aorta. When aneurysmal dilatation was elicited with periaortic application of $CaCl_2$ and thioglycollate, macrophages were the dominant inflammatory cell recruited into the aorta, with a scattering of lymphocytes and occasional giant cells in calcified areas.[20] There was no significant increase in the number of macrophages (27 ± 15 per unit area) or lymphocytes after aortas had been treated with heat-inactivated *C pneumoniae* in $CaCl_2$. After aorta had been treated with live or formalin-inactivated *C pneumoniae* (5×10^7 organisms), the number of macrophages increased significantly to 324 ± 144 and 143 ± 69 and per unit area, respectively (ANOVA, $P < .01$), and there was a modest increase in lymphocytes. At a lower dose (5×10^5) of formalin-inactivated organism, macrophages (53 ± 19 per unit area) were confined largely to the media, but at even lower doses

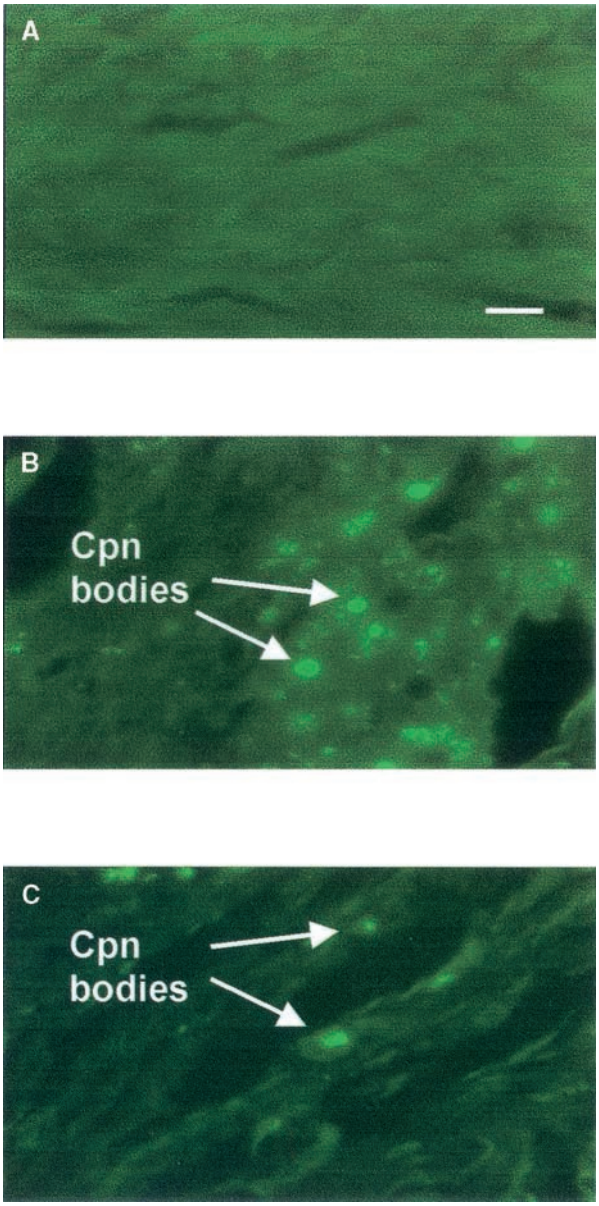

Fig 1. *C pneumoniae* elementary bodies visualized with antibody TT-401. Sections of formalin-fixed tissue have been stained with fluorescein-conjugated TT-401 (1 in 100 dilution), and *scale bar* represents 10 μm. *Panel A* shows control aorta, 3 weeks after periaortic application of 0.2 mol/L $CaCl_2$ only. There is only background fluorescence. *Panel B* shows aorta of rabbit, 3 weeks after periaortic application of formalin-inactivated *C pneumoniae* (5×10^7 organisms) in 0.2 mol/L $CaCl_2$. High density of elemental bodies is evident. *Panel C* shows aorta from rabbit treated as in *panel B* but given a 10-day course of azithromycin. Density of elemental bodies is reduced compared with *panel B*.

of organism (5×10^3 or 5×10^1), negligible macrophage staining (28 ± 18 and 19 ± 15 per unit area, respectively) or lymphocytes were observed.

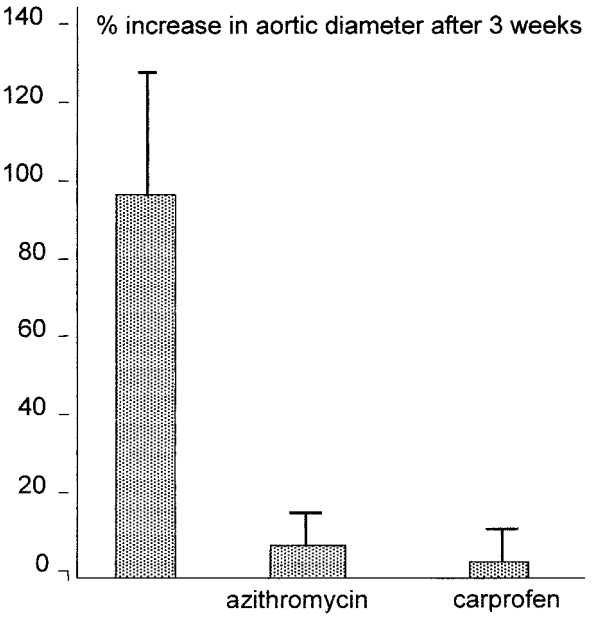

Fig 2. Effect of azithromycin and carprofen on aortic dilatation provoked by periaortic application of formalin-inactivated *C pneumonia*. Results show mean (standard deviation) of percentage increase in aortic diameter, with four animals in each group. Treatment with either azithromycin or carprofen abolished increase in aortic diameter (ANOVA, $P < .02$).

Intervention with azithromycin or carprofen. Rabbits (n = 12) were randomly allocated to receive a 10-day postoperative course of azithromycin, carprofen (a nonsteroidal antiinflammatory drug suitable for use in rabbits), or no treatment: formalin-inactivated *C pneumoniae* (5×10^7 organisms) in $CaCl_2$ was applied to the abdominal aorta. The increase in aortic diameter, measured after 3 weeks, is shown in Fig 2: only the animals with no drug treatment showed an increased aortic diameter (ANOVA, $P < .01$). Representative immunostaining for macrophages, with RAM-11, is shown in Fig 3. In the no treatment group, there was evidence of medial calcification, disruption of normal tissue architecture, and a transmural infiltrate of inflammatory cells, predominantly macrophages. After treatment with azithromycin, aortic calcification was still evident, but only a few macrophages surrounding the calcified areas were observed. Similarly, the number of elemental bodies detected with staining with TT-401 was markedly diminished (Fig 1 , *panel C*). After treatment with carprofen, medial aortic calcification with a considerable number of macrophages and scattered lymphocytes was still evident, but the inflammatory cells were confined principally to the media. The macrophage counts/unit area in the no treatment, carprofen, and azithromycin groups were 158 ± 44, 109 ± 5, and 3 ± 4, respectively (ANOVA, $P < .01$).

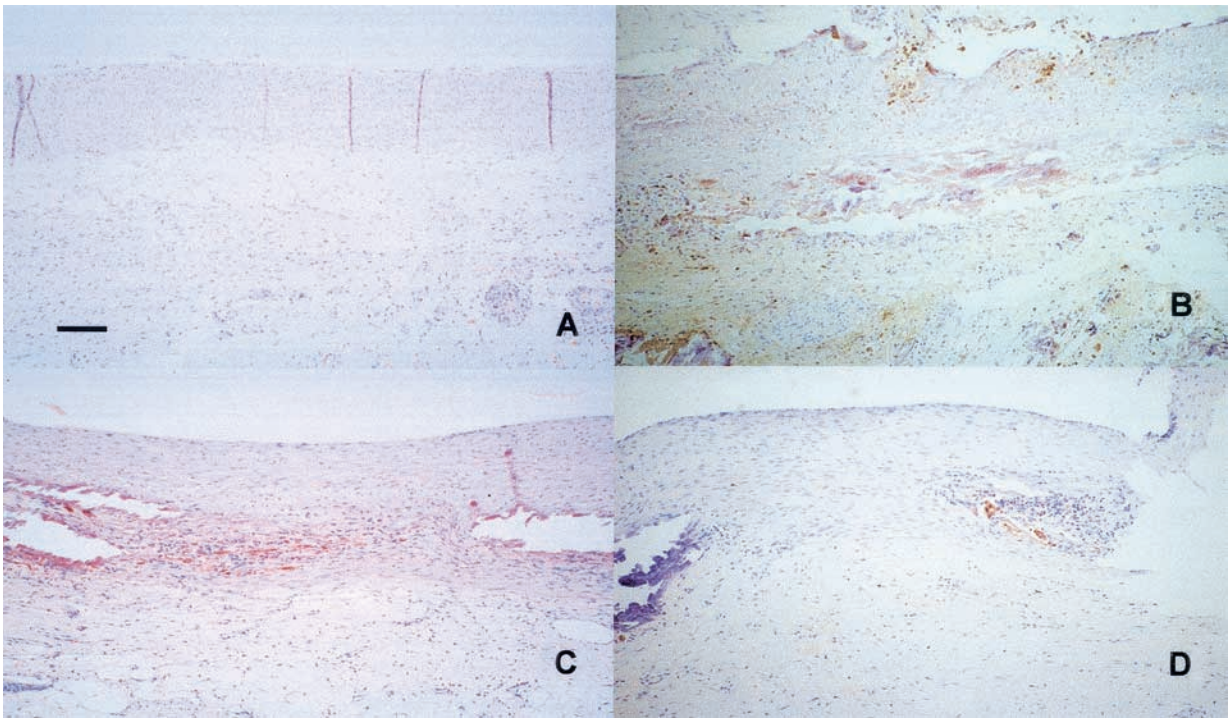

Fig 3. Aortic macrophages visualized with antibody RAM-11. Formalin-fixed sections have been stained with RAM-11 (*brown*) and counterstained with hematoxylin; *scale bar* represents 50 μm. *Panel A* shows normal rabbit aorta, 3 weeks after periaortic application of NaCl. *Panel B* shows rabbit aorta 3 weeks after periaortic application of formalin-inactivated *C pneumoniae* (5×10^7 organisms) in 0.2 mol/L $CaCl_2$. Tissue architecture is disrupted, with evidence of medial calcification and intense transmural infiltrate of macrophages. *Panel C* shows aorta from rabbit treated as in *panel B* but given 10-day course of carprofen. There is still evidence of medial calcification, but fewer macrophages mainly confined to media near areas of calcification. *Panel D* shows aorta from rabbit treated as in *panel B* but given 10-day course of azithromycin. There is still evidence of medial calcification but only scant presence of macrophages.

DISCUSSION

Our initial results showing that the periaortic application of live *C pneumoniae* provoked aortic dilatation suggested possible proof of Koch's third postulate for AAA; further experiments disproved this possibility. Live organisms were not essential to provoke aortic dilatation, and similar results were obtained with inactivated organisms with antigen preservation (formalin-inactivated organisms). For both live and formalin-inactivated organisms, there was persistence of chlamydial antigens in the aortas harvested at 3 weeks. In contrast, preservation of lipopolysaccharide with denaturation of antigens (heat-inactivated organisms) did not provoke an increase in aortic diameter, providing confirmation of previous work showing that bacterial lipopolysaccharide did not provoke aortic dilatation (Walton LJ, unpublished observations). Moreover, with heat-inactivated preparations, no organisms could be detected in the aorta after 3 weeks. Therefore, it appears that chlamydial antigen persistence, rather than infection, was associated with the aortic dilatation in this model.

For safety reasons, we pursued most of our further investigations with formalin-inactivated preparations of *C pneumoniae*. Treatment with either azithromycin or a nonsteroidal antiinflammatory drug (carprofen) proved effective in preventing the aortic dilatation stimulated by formalin-inactivated organisms.

Azithromycin is the drug previously used to retard the development of atherosclerosis in rabbits and in human prevention trials.[16-18,21] Azithromycin is recognized to have antiinflammatory and antimacrophage activity, and this alternative mode of drug action must be considered.[17,22] Recent evidence also shows that azithromycin improves endothelial function in patients with evidence of previous *C pneumoniae* infection.[23] Therefore, azithromycin might improve the endothelial dysfunction resulting from periaortic application of calcium chloride.[20]

In this experimental model, aortic dilatation always is accompanied by a heavy transmural influx of macrophages.[20] Chlamydial antigens and thioglycollate (a nonspecific macrophage activator) appeared to provoke a similar magnitude of macrophage influx. Carprofen prevented aortic dilatation but had only a marginal effect on the number of macrophages in aorta. Carprofen will inhibit cyclooxygenase 2 and through this mechanism is likely to

inhibit the formation of inflammatory prostanoid mediators. In man, and animal models, there is evidence to show that inhibition of cyclooxygenase 2 slows aneurysm growth and reduces the production of inflammatory cytokines in the aorta.[24,25] Both macrophage recruitment and macrophage activation are likely to be necessary to cause aortic dilatation in our experimental model. In addition, our study confirms that azithromycin, at least in rabbits, has specific antimacrophage effects. A similar mechanism may underlie the early beneficial clinical effects of azithromycin that have been reported.[21]

There has been considerable discussion as to whether there is an infectious basis for atherosclerosis. Recent evidence from studies with apolipoprotein E knockout mice shed doubt on this possibility.[26,27] However, there has also been considerable discussion of the other potential mechanisms of how *C pneumoniae* could aggravate atherosclerosis through antigen mimicry and immune mechanisms, and the persistence of chlamydial antigens in AAA tissue has been recognized.[28-30] Our findings are in keeping with such mechanisms.

Inflammation and proteolysis are the twin processes considered to underlie aortic dilatation, with matrix metalloprotease-9 being considered as one of the pivotal proteolytic enzymes involved in aneurysm expansion in man and experimental animals.[10,31,32] The chlamydial membrane antigens omp-2 and major outer membrane protein and the secreted heat shock protein-60 stimulate the secretion of matrix metalloprotease-9 from human macrophages in vitro.[33] A role for chlamydial antigens in compounding other risk factors for aneurysmal disease is in keeping with the findings of Meijer et al,[14] who reported that membrane antigens, specific to *C pneumoniae*, were found in all samples of AAA, even in the absence of DNA and heat shock protein. In our experimental model, such membrane antigens could be responsible for the inflammatory response, with macrophage recruitment and activation.

Strengths of our study include the formal testing of Koch's third postulate and the use of active and inactivated organisms, but there are several limitations. First, although the experimental model has histologic similarities with the human disease, the conditions required to cause rapid aneurysmal dilatation are extreme: an inflammatory stimulus in the presence of high serum cholesterol concentrations and sufficient calcium chloride to cause endothelial dysfunction, transmural injury, and calcification.[20] Similar extreme conditions also cause carotid artery aneurysms in rabbits.[34] Second, large inoculates of formalin-inactivated *C pneumoniae* were required to cause aortic dilatation, although similar size inoculates were necessary to potentiate atherosclerosis in cholesterol-fed rabbits.[16] Third, much larger groups of animals would have been necessary to show a dose-dependent effect on aortic diameter increase. Fourth, the small number of animals in each experimental group (in accord with current ethical guidelines) permits only the detection of large effects.

In summary, the evidence from this experimental model indicates that infection with *C pneumoniae* could accentuate antigen-dependent inflammatory pathways in the aortic wall to contribute to experimental AAA development. However, such extrapolation from rabbit to man must be made with extreme caution. Although antigen-dependent inflammatory pathways are likely to contribute to aneurysm development, there is still no evidence that infection with *C pneumoniae* causes AAA.

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